

Rapid Transmethylation of Microgram Amounts of Phosphatidylcholine on Potassium Methoxide/Celite Columns^{1,2,3}

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ABSTRACT

A rapid and convenient method for the determination of acyl groups in phosphatidylcholine (PC) has been developed. Transmethylation reactions were carried out on potassium methoxide-impregnated Celite microcolumns that were readily prepared from Pasteur pipettes. The methods were tested on microgram amounts of synthetic L- α -phosphatidylcholines (di-14:0; di-18:2; α -16:0- β -14:0) and on egg yolk lecithin, in methylene chloride or hexane solution. Transmethylation of these lipids occurred rapidly at room temperature. Gas liquid chromatographic (GLC) analysis of the product methyl esters demonstrated that the reaction was neither selective for one acyl position of PC over the other, nor sensitive to the amount of unsaturation within the acyl group. The results of the acyl group analysis of natural egg yolk lecithin compared favorably with the results from an established procedure.

INTRODUCTION

This paper addresses the analysis of the acyl groups of phospholipids and, in particular, the largest subclass, phosphatidylcholine (PC). In standard procedures (1-3), methyl esters for gas liquid chromatographic analysis (GLC) are generated from milligram amounts of PC in methanol in the presence of basic or acidic catalysts. Especially rapid transesterification occurs in a solution of sodium or potassium methoxide in methanol (4-6). The ester products then are extracted from the reaction medium, concentrated by solvent evaporation, and finally injected onto a GLC column.

In earlier research with neutral lipids, Schwartz demonstrated the advantages of derivatization via microcolumn procedures (e.g., 7-10), including the feasibility of transmethylation of microgram amounts of triglycerides on a potassium methoxide/Celite microcolumn (11). The present research was undertaken to (a) expand upon the microcolumn transmethylation technique, (b) refine the microcolumn preparation procedure in order to facilitate column packing and impart long term stability and storability, and (c) demonstrate the effectiveness of the technique with regard to polar lipids and, particularly, PC. The following results with PC demonstrate the con-

venience and simplicity of this transmethylation method. A newly developed, prepacked and disposable microcolumn need only be snapped open and treated with a few micrograms of lipid, such as isolated from a thin layer chromatographic (TLC) spot, and then eluted of product esters for GLC analysis.

EXPERIMENTAL PROCEDURES

Reagent on Celite

Celite diatomaceous earth (6.0 g analytical grade, Fisher Scientific Co., Pittsburgh, PA) was ground thoroughly and rapidly with powdered potassium methoxide (1.0 g, Ventron Corp., Danvers, MA). The resulting mixture was transferred into a screw cap vial.

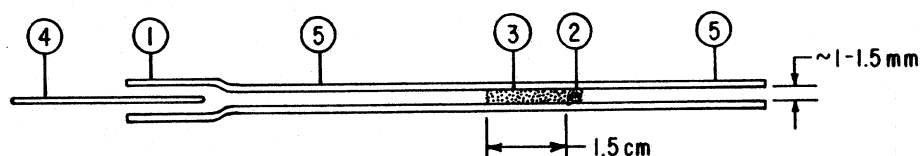
Column Preparation

The Schwartz procedure (11) was modified to simplify column preparation and to maintain reagent activity over prolonged storage conditions. The new procedure is outlined in Figure 1. A glass capillary (i.d. 11.5 mm) with an attached funnel was obtained by shortening a 9 in. Pasteur pipette. The residual wide end was sealed at one end and used later as a disposable collection vessel for column eluate. A small plug of glass wool was inserted into the capillary via the funnel end by means of forceps and a wire tamper. Discarded plungers from microliter syringes were convenient tampers. Anything introduced into the capillary must not be contaminated with fingerprints, which are a rich source of lipids in microgram quantities. The plugged capillaries were dried in an oven at 120 C and then transferred into a dry

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²Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

³Chloroform and carbon disulfide have been classified by OSHA as carcinogens and/or toxic materials; they should be handled with care under proper ventilation.



- ① PASTEUR PIPETTE, SHORTENED
- ② GLASS WOOL PLUG
- ③ REAGENT ON CELITE
- ④ WIRE TAMPER
- ⑤ LOCATION OF SEALING FOR STORAGE OR HIGH T REACTION

FIG. 1. Microcolumn preparation.

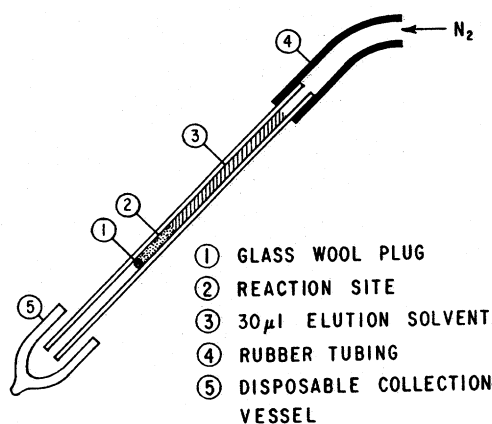


FIG. 2. Elution of reaction products.

box for subsequent packing. Sufficient amounts of the reagent/Celite mixtures were added via the funnel ends so that a 1.5 cm column resulted after subsequent tamping with the wire plunger. Finally the dry columns were removed from the dry box and immediately sealed at both ends by means of a small flame. The resulting sealed columns (9 cm long) were stored in vials at room temperature for future use.

Lipid Solutions

The following synthetic L- α -phosphatidylcholines were used as model compounds: α -palmitoyl- β -myristoyl- (Supelco Inc., Bellefonte, PA), dimyristoyl- (Sigma Chemical Co., St. Louis, MO), and dilinoleoyl- (PL Biochemicals, Inc., Milwaukee, WI). Purity was ascertained by silica gel TLC using each of two solvent systems (chloroform³-methanol-acetic acid-water, 25:15:4:2 and chloroform-

methanol-ammonium hydroxide 10:5:2, by volume). Egg yolk lecithin (Sigma Chemical Co., St. Louis, MO) served as a natural PC. Solutions of these lipids were prepared in methylene chloride or hexane (Nanograde, Mallinckrodt, Inc., St. Louis, MO) to contain 2.5 $\mu\text{g}/\mu\text{l}$. Individual solutions of (14:0)₂ PC and (18:2)₂ PC were analyzed for their lipid content by quantitative determination of their phosphorus content (12). These solutions then were combined to give a new solution with equal weights of each lipid.

Potassium Methoxide Reactions

A prepacked potassium methoxide/Celite microcolumn was scored and snapped open at both ends. The PC solution (1 to 10 μl) was introduced with a 10 μl syringe. The open column ends then were sealed temporarily with Critoseal vinyl plastic putty (made for sealing microhematocrit tubes, A.H. Thomas Co., Philadelphia, PA). Two to 6 min later the putty-sealed ends were scored and snapped off, and the methyl esters were eluted with methylene chloride or carbon disulfide (30 μl), as depicted in Figure 2. Elution was expedited by application of nitrogen under pressure. Prolonged contact with the potassium methoxide led to reduced yields, probably due to slow concomitant saponification (13). The eluate was collected in a small disposable vessel made from the cut-off wide end of a Pasteur pipette or in some cases simply removed from the lower end of the column with a 10 μl syringe. The eluate was then introduced into the GLC inlet. Prior concentration by evaporation was avoided (*cf.* below). In the case of one 2 min reaction, the column was acidified by drawing hydrogen chloride vapor over it, then eluted

TABLE I
Transmethylation of Synthetic PC by Microcolumn^a

Reaction	Normalized values			Standard deviation
	14:0	16:0	18:2	
α -16:0- β -14:0 in CH ₂ Cl ₂ , 6 min, 25 C	47.0	53.0	---	± 1.5
Calculated FID response ^b	46.7	53.3	---	---
Equal weights of (14:0) ₂ PC and (18:2) ₂ PC in hexane, 2 min, 25 C	48.6	---	51.4	± 1.3
Calculated FID response ^b	48.8	---	51.2	---

^a25 μ g in 10 μ l solvent.

^bFlame ionization detector, cf text (14,15).

TABLE II
Transmethylation of Egg Yolk Lecithin by Microcolumn

Ester ^a	Normalized values by		Method of Luddy et al. ^c
	KOMe/Celite method ^b		
16:0	33.16 \pm 0.61		33.29 \pm 0.53
16:1	1.18 \pm 0.10		1.12 \pm 0.06
18:0	14.59 \pm 1.41		13.72 \pm 0.73
18:1	28.69 \pm 1.27		30.54 \pm 0.24
18:2	15.58 \pm 0.73		15.94 \pm 0.18
20:4	4.58 \pm 0.82		3.75 \pm 0.29
Unidentified	1.18 \pm 0.18		0.75 \pm 0.13
22:6	1.12 \pm 0.09		0.90 \pm 0.19

^aIn order of appearance.

^bMean \pm standard deviation for 4 runs @ 30 μ g lipid, 6 min, 25 C.

^c1 run @ 2 mg lipid, 4 aliquots for GLC (5).

with methylene chloride and then methanol. TLC analysis of the eluate showed the absence of PC and its monoacylated hydrolysis product, lysophosphatidylcholine.

Gas Liquid Chromatography

GLC separations of methyl myristate from either methyl palmitate or methyl linoleate were achieved on a Hewlett-Packard 5750 instrument equipped with a flame ionization detector (FID). An EGA/phosphoric acid column was used (7.5% stabilized ethylene glycol adipate and 2% phosphoric acid on 90-100 mesh Anakrom ABS; 8 ft by 1/8 in. silanized stainless steel). The column was maintained at 130 C for 4 min and then programmed up to 200 C at 6°/min. Separation of the complex mixtures of methyl esters derived from transmethylation of egg yolk lecithin was done on a Perkin-Elmer Sigma 3 gas chromatograph in the FID mode. In that case, separations were achieved on a wall-coated, open tubular, 50 meter glass capillary column with a stationary phase of diethylene glycol succinate (Perkin-Elmer No.009-7765). The column was

maintained at 140 C for 4 min and then programmed up to 190 C at 4°/min. Peak areas were determined with a Supergrator 2 programmable computing integrator (Columbia Scientific Industries, Austin, TX). Integral values of FID-generated signals were considered to be proportional to the number of noncarbonyl carbon atoms; for long chain fatty esters, these values are approximately proportional to the mass (14,15). Retention times were compared to those of standard esters (Nu-Chek Prep., Inc., Elysian, MN).

Evaporation Experiment

The α -16:0- β -14:0 PC (25 μ g) in methylene chloride (10 μ l) was allowed to react on a potassium methoxide/Celite microcolumn in the usual way. The eluate was collected, and an aliquot was immediately withdrawn and analyzed by GLC. The remainder was exposed to a constant stream of nitrogen; at intervals of 2, 4, and 6 min, the solution was reconstituted by addition of methylene chloride, and aliquots were injected onto the GLC column.

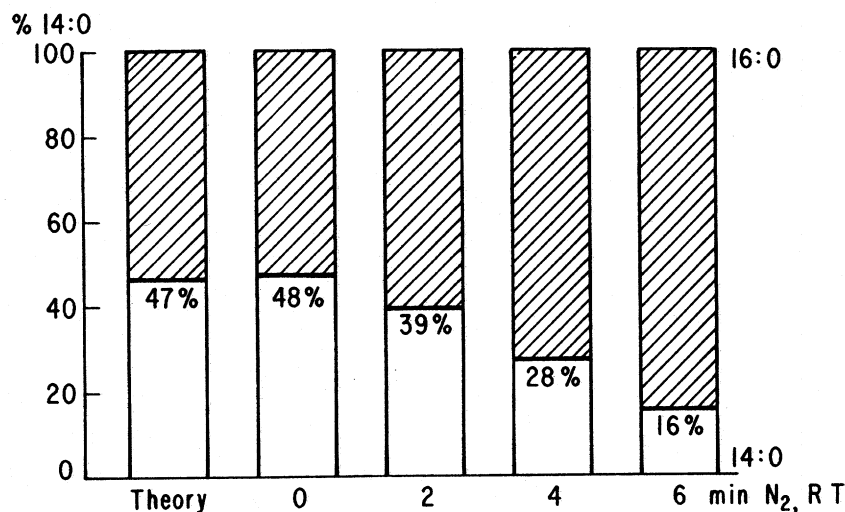


FIG. 3. Effect of N₂ evaporation on GLC results. (Reaction of 25 mg model PC on KOMe/Celite. Bars illustrate normalized ratios of 14:0/16:0 detected by GLC. Percentage of 14:0 within bars.)

RESULTS AND DISCUSSION

Seven samples of the α -16:0- β -14:0 PC (6 x 25 μ g, 1 x 2.5 μ g) and five samples of the (14:0)₂ PC/(18:2)₂ PC equal weight mixture (5 x 25 μ g) were analyzed for their total acyl group content by reaction on potassium methoxide/Celite microcolumns. Results are given in Table I. The data are in good agreement with the expected values, and demonstrate a lack of selectivity for saturated vs. unsaturated acyl groups and for α -acyl vs β -acyl groups.

Five samples of egg yolk lecithin (30 μ g in 10 μ l CH₂Cl₂) were analyzed for acyl content after transmethylation on potassium methoxide/Celite microcolumns. The results are listed in Table II and correlate well with data obtained by use of the procedure of Luddy et al. (5) on 2 mg of the same lipid.

All results were substantially altered whenever the eluate of esters was subjected to evaporation under vacuum or nitrogen stream. To illustrate the effect of the nitrogen stream, the eluate from a single reaction of 25 μ g of the α -16:0- β -14:0 PC on a potassium methoxide/Celite microcolumn was sampled prior to evaporation and then after 2, 4, and 6 min under a constant nitrogen stream at room temperature. Results are illustrated in Figure 3. The severity of product loss through selective evaporation of these methyl esters was unexpected, though Ackman and Burgher (16) warned of moderate losses of 14:0 over 16:0 during evaporation of milligram amounts of mixtures. Indeed, a call for avoidance of such

evaporation would be contrary to several published methods (1-3). The selective evaporation of 14:0 over 16:0 was, to be sure, exaggerated at the microgram level and was even noticed in samples that were exposed only to room air currents. The problem was obviated by injection of aliquots of the unevaporated eluate directly into the GLC inlet at a relatively low column temperature. After dissipation of the large solvent peak, the esters were allowed to elute by a subsequent programmed increase in column temperature.

Another problem involved the use of chloroform as the solvent during reaction. The 0.75% ethanol that is typically added to chloroform by the manufacturer as a stabilizer was sufficient to give rise to considerable amounts of ethyl ester byproduct. This may be understood better by comparing the molar amount of methoxide in a typical microcolumn (13 μ mol) with the molar amount of ethanol in 10 μ l of lipid solution (2.4 μ mol). For each μ mol of ethoxide, there are only 5.5 μ mol of methoxide. A related problem recently was reported in the literature (17).

The data in Tables I and II demonstrate that by heeding the warnings concerning fingerprints, evaporation, and chloroform, one may use the potassium methoxide microcolumn procedure to establish the acyl composition of phosphatidylcholine. Standard deviation calculations on repetitive runs demonstrate, furthermore, that the results are reproducible. The advantages gained by use of this new procedure are, of course, amplified when multiple analyses are required.

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REFERENCES

1. Anon., "Official and Tentative Methods of the American Oil Chemists' Society," 3rd ed., Method Ce 2-66, Champaign, IL, AOCS, 1966, revised 1969.
2. Christie, W.W., in "Lipid Analysis," Pergamon Press, Oxford, 1973, p. 87ff.
3. Kates, M., "Laboratory Techniques in Biochemistry and Molecular Biology," Vol. 3, Part II, Edited by T.S. Work and E. Work, North Holland Publishing Co., Amsterdam, 1972, p. 361ff.
4. Sheppard, A.J., and J.L. Iverson, *J. Chromatogr. Sci.* 13:448 (1975).
5. Luddy, F.E., R.A. Barford, S.F. Herb, and P. Magidman, *J. Am. Oil Chem. Soc.* 45:549 (1968).
6. Christie, W.W., *Analyst* 97:221 (1972).
7. Schwartz, D.P., and J.L. Weihrauch, *Microchem. J.* 18:249 (1973).
8. Schwartz, D.P., and R.S. Bright, *Anal. Biochem.* 61:271 (1974).
9. Schwartz, D.P., *Anal. Biochem.* 71:24 (1976).
10. Schwartz, D.P., *Anal. Biochem.* 74:320 (1976).
11. Schwartz, D.P., *Microchem. J.* 22:457 (1977).
12. Vaskovsky, V.E., E.Y. Kostetsky, and I.M. Vasendin, *J. Chromatogr.* 114:129 (1975).
13. Glass, R.L. *Lipids* 6:919 (1971).
14. Ackman, R.G., "Methods in Enzymology," Chapter 49, Volume XIV, Edited by J.M. Lowenstein, Academic Press, New York, 1969, p. 339ff.
15. Ackman, R.G., in "Progress in the Chemistry of Fats and Other Lipids," Vol. 12, Edited by R.T. Holman, Pergamon Press, Oxford, 1972, p. 254ff.
16. Ackman, R.G., and R.D. Burgher, *J. Lipid Res.* 5:130 (1964).
17. Johnson, A.R., A.L. Fogerty, R.L. Hood, S. Kozuharov, and G.L. Ford, *J. Lipid Res.* 17:431 (1976).